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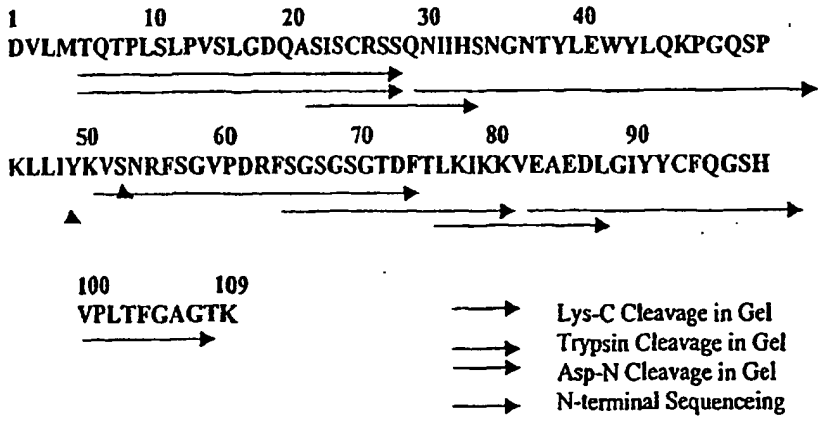
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(54) Title: METHODS AND COMPOSITIONS FOR DISEASES ASSOCIATED WITH AMYLOIDOSIS



(57) Abstract: The present invention generally relates to the detection, treatment or prevention of disease states. Specifically, the present invention relates to the detection, treatment or prevention of amyloidosis or amyloid-associated diseases. The present invention further comprises methods and compositions comprising therapeutic vaccines, antisera and molecular constructs, comprising expression vectors and fusion proteins encoded therein.

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METHODS AND COMPOSITIONS FOR DISEASES ASSOCIATED WITH AMYLOIDOSIS

FIELD OF THE INVENTION

The present invention generally relates to the detection, treatment or prevention of disease states. Specifically, the present invention relates to the detection, treatment or prevention of amyloidosis or amyloid-associated diseases.

BACKGROUND OF THE INVENTION

Amyloidosis includes a variety of diseases characterized by an accumulation of amyloid material in the organs or tissues of the body. This accumulation can impair vital functions. Diseases associated with amyloidosis include Alzheimer's disease (AD), Down's Syndrome, progressive supranuclear palsy, multiple sclerosis, and Adult Onset Diabetes. Localized amyloidosis is associated with cognitive decline (senile cerebral amyloidosis; Alzheimer's), heart disease (senile cardiac amyloidosis), endocrine tumors (thyroid cancer), and Adult Onset Diabetes, diseases which are found in millions of Americans.

A number of impairments specific to amyloid deposits in the brain are linked with the deposition of the peptide, Aβ peptide (amyloid-β peptide). Neurological diseases associated with Aβ peptide deposition include Alzheimer's, Lewy body dementia, Down's Syndrome, hereditary cerebral hemorrhage with amyloidosis (Dutch type), and the Guamanian Parkinsonism-Dementia. Aβ peptide plaques also occur in persons who have experienced head trauma and critical coronary disease.

The most common disease related to cognitive decline or dementia is Alzheimer's Disease (AD). This condition is characterized by neuronal loss, neurofibrillary tangles, and neuritic plaques comprised of Aβ peptide. Due to

the nature of cerebral amyloidosis, diagnosis of Alzheimer's before death is difficult and the development of therapies or other treatments for Alzheimer's have been elusive. Many proposed therapies are unable to cross the blood-brain barrier in amounts necessary for effective treatment.

It is currently believed that amyloid plaques in the brain are predominantly composed of Aβ peptide, a 4 kD protein, 39-43 amino acids long. Aβ peptide is expressed by a gene located on chromosome 21 and is derived by proteolytic cleavage from the interior of a much larger (770 residue) cell protein, amyloid precursor protein (APP). After excision, Aβ peptide is polymerized into amyloid filaments, which in turn aggregate into amyloid plaque deposits. In the brain, these filaments and aggregates are toxic to neurons and are thought to relate to the causative symptoms associated with AD.

The inability to examine amyloid deposition of AD in patients before death impedes the ability of researchers to study AD and develop effective therapies targeted at preventing or reversing amyloid plaque formation on the brain. Damage to CNS neurons due to AD begins years before clinical symptoms are evident. Prevention of amyloidosis in the brain would prevent the development of AD. A similar approach to preventing or treating amylin plaque formation in Adult Onset Diabetes patients would also be beneficial.

Other investigations have identified a set of mutations that cause familial Alzheimer's disease (FAD), in an autosomal dominant manner. All known FAD mutations increase either the absolute or relative production of the most pathogenic Aβ peptide with 42 amino acids. These mutations arise either in the APP gene itself, affecting the target sites in the APP where proteolytic cleavage occurs, or in one of two presenilin genes, the protein products which directly or, more probably, indirectly affect APP processing. The early step of AD excision is followed by the polymerisation of the peptide to amyloid filaments, which in turn aggregate into the visible amyloid plaque

deposits that characterize the brains of individuals with AD. These filaments, or possibly intermediate protofilamants, are toxic to neurons and are thought to lead to neurofibrillary tangles, synapse loss, and neurodegeneration that underlie the decline of cognitive functions in Alzheimer's patients.

In addition to the production of the Aβ peptide (amyloid-β peptide), other important steps are involved in the pathogenic pathway leading to AD. Genetic and biochemical studies both assign a key role to a set of auxiliary proteins that function as "pathological chaperones". One such protein is the anti-protease, anti-chyrmotrypsin, and another is the lipid transport protein, apolipoprotein F, particularly its E4 allelic form. Both of these proteins promote the formation and maturation of Alzheimer's plaques with their core of Aβ filaments. Specifically, the pathological chaperones can be demonstrated in vitro to accelerate the polymerisation of Aβ into neurotoxic amyloid filaments, and experiments with transgenic mice support their role in promoting amyloid formation in vivo. Both proteins are minor components of the amyloid deposits, and are evidently produced as part of an inflammation reaction that arises in response to the initial diffuse deposition of Aβ peptide.

What is needed are methods and compositions for diagnosis and treatment of amyloid-associated diseases.

SUMMARY OF THE INVENTION

The present invention is directed to compositions and methods for diagnosis, prevention and treatment of disease states associated with amyloidosis. In accordance with the present invention, compositions and methods are provided that are effective in detecting and treating amyloid deposition, especially amyloid deposition related to neurodegenerative diseases. In a preferred aspect, amyloid deposition is detected and treated by compositions comprising a fusion protein, comprised of a segment comprising an antibody or antibody fragment that binds to an amyloid peptide epitope and

a segment capable of crossing the blood-brain barrier, such as one or more transferrin moieties. Preferably, the fusion protein composition has amyloid plaque or tangle dissolving properties and is capable of crossing the blood-brain barrier.

Specifically, the present invention provides for the construction and use of a fusion protein that can detect and treat β-amyloid deposition. A preferred fusion protein is comprised of an anti-beta-amyloid monoclonal antibody binding region and one or more transferrin moieties. The present invention comprises molecular constructs such as nucleic acid vectors, proteins expressed by such vectors and cells transformed with such vectors and that express the proteins coded for by such vectors.

The present invention also comprises methods and compositions comprising a vaccine that elicits an immune response against amyloid proteins, peptides or fragments, and prevents, stops or hinders amyloid deposition. The vaccine is comprised of an antigen, an amyloid peptide fragment or epitope that is preferably provided in a liposomal bilayer. In an embodiment of the present invention, an antigen is comprised of a modified amyloid peptide, preferably palmitoylated β-amyloid$_{1-16}$ peptide, and methods of administration in a liposomal bilayer.

The invention also provides a method and compositions for preventing or alleviating the symptoms of disease states associated with accumulation or molecular organization of amyloid protein or amyloid plaques in general, including the administration of a pharmaceutically effective amount of a derivaterized amyloid peptide or peptide fragment to a patient.

The present invention includes a pharmaceutical composition in an amount effective to prevent, stop or impede one or more symptoms of a disease state involving abnormal accumulation or molecular organization of amyloid protein, assemblies, fibrils, filaments, tangles, or plaque deposits. The present invention includes an antigen to stimulate production of anti-

amyloid monoclonal antibody with the ability to dissolve amyloid plaques. The present invention also provides a method for treating a disease state associated with amyloidosis, including the administration of a pharmaceutically effective amount of the vaccine to a patient.

The present invention also comprises methods for making compositions comprising antisera raised to the epitope of the vaccine compositions of the present invention. These antisera compositions are capable of dissolving amyloid aggregates comprising the epitope or antigen (e.g. β-amyloid). The antisera compositions are effective in the treatment or prevention of the accumulation amyloid protein or the formation of amyloid assemblies, fibrils, filaments, tangles, or plaque deposits and, therefore, disease states associated with amyloidosis.

The invention further comprises methods and compositions for preventing or alleviating the symptoms of disease states associated with accumulation or molecular organization of amyloid protein or amyloid plaques including the administration of a pharmaceutically effective amount of antisera to a patient.

The present invention is particularly useful in the treatment of amylodi-associated diseases, preferably, β-amyloid-associated diseases, including Alzheimer's Disease, Lewy body dementia, Down's Syndrome, hereditary cerebral hemorrhage with amyloidosis (Dutch type), and the Guam Parkinson-Dementia complex or other diseases involving abnormal accumulation or molecular organization of amyloid protein, assemblies, fibrils, filaments, tangles, or plaque deposits.

This and other objects, features, and advantages of the present invention will become apparent after a review of the following detailed description of the disclosed embodiments and the appended claims.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 Sequence of Vl chain of 6C6 with sequence sites.

FIG. 2(a)is a map of vector pET24d+.

FIG. 2(b) is a linear map of vector pET24d+

FIG. 3 is a map of vector pET36.

FIG. 4(a) shows the map of the pBlueBacHIS2 B vector containing the V_L fragment.

FIG. 4(b) shows the map of the pBlueBacHIS2 B vector containing the V_L-transferrin fragment.

FIG. 5 is the ß-amyloid l- 16 ($Aß_{1-16}$) peptide anchored to the liposomal bilayer by palmitoylated lysines bound to both ends of the Aβ 1-16 peptide.

FIG. 6(a) Synthetic Pathway of dipalmitoyllysine using direct palmitoylation described in Examples 2 and 3.

FIG. 6(b) Synthetic Pathway of dipalmitoyllysine using activated esthers described in Examples 2 and 3.

FIG. 6(c) Solid Phase Synthetic Pathway of the C-Terminus Described in Example 3.

FIG. 7 is a FAB-mass spectrum of the palmitoylester of N-hydroxysuccinimide. The expected peak at 623 Da (MH+) and absence of the peak for the activated ester (354 Da) are visible.

FIG.8 is a ^{1}H NMR spectrum of the palmitoylester of N-hydroxysuccinimide. The expected signals are visible.

FIG. 9 depicts the disaggregation of ß-amyloid fibers by the 6C6 = mAB and by sera from mice immunized with palmitoylated Aß (1-16) reconstituted in liposomes.

DETAILED DESCRIPTION

The present invention is directed to compositions and methods for diagnosis, treatment and prevention of diseases associated with amyloid deposition. Preferred compositions of the present invention include compositions comprising fusion proteins capable of affecting amyloid deposits that can cross the blood-brain barrier, and compositions that are effective in raising an immune response to epitopes of amyloid deposits. Preferred methods of the present invention comprise use of the compositions for diagnosis, treatment and prevention of diseases associated with amyloid deposition, and for making and using modified amyloid peptides and molecular constructs of amyloid genes.

An example of one of the diseases associated with amyloid deposition is Alzheimer's Disease, AD. By definition, AD is diagnosed through the examination of brain tissue, usually at autopsy. The currently recommended "minimum microscopic criteria" for AD diagnosis is based on the number of neuritic plaques found in the brain. The amyloid cores of these neuritic plaques are composed of ß-amyloid protein, also referred to herein as amyloid- ß peptide, that is arranged in a predominately beta-pleated sheet

configuration. Brain amyloid plaques are demonstrated by staining brain sections with thioflavin S or Congo red. Congo red-stained amyloid is characterized by a dichroic appearance, exhibiting a yellow-green polarization color. The dichroic binding is the result of the beta-pleated sheet structure of the amyloid proteins. .

It is very difficult to diagnose Alzheimer's disease before death, to develop drug therapies, or to treat AD. Screening for apolipoprotein E genotype has been suggested as an aid in the diagnosis of AD. Difficulties arise with this technology, however, because the apolipoprotein E4 allele is only a risk factor for AD, not a disease marker. It is absent in many AD patients and present in many non-demented elderly people. Immunoassay methods have been developed for detecting the presence of neurochemical markers in AD patients and to detect an AD-related amyloid protein in cerebral spinal fluid. These methods for diagnosing AD have not been proven to detect AD in all patients, particularly at early stages of the disease. They are also relatively invasive, requiring a spinal tap. Recently, radiolabeled Aβ peptide has been used to label diffuse, compact and neuritic type plaques in sections of AD brain. These peptides, however, do not normally cross the blood-brain barrier in amounts necessary for imaging.

Congo red may be used for diagnosing amyloidosis *in vivo* in non-brain parenchymal tissues. But Congo red is probably not suitable for *in vivo* diagnosis of ß-amyloid deposits in brain because only very small amounts, approximately 0.03% of an injected dose of iodinated Congo red, can enter the brain parenchyma. Radioiodinated bisdiazobenzidine compounds related to Congo red, such as Benzo Orange R and Direct Blue 4, have been proposed to be useful *in vitro* and *in vivo* to detect the presence and location of amyloid deposits in an organ of a patient. However, like Congo red, all of the compounds contain strongly acidic sulfonic acid groups which severely limit entry of these compounds into the brain.

Attempts have also been made to develop monoclonal antibodies as probes for imaging of amyloid plaques. For instance, antibodies raised against the N-terminal region (1-28) of ß-amyloid bind to *in vitro*-formed β-amyloid assemblies, leading to disaggregation of fibrils and partial restoration of ß-amyloid solubility. Some of the monoclonal antibodies raised against soluble ß-amyloid (1-28) have also been found to inhibit fibrillar aggregation of β-amyloid peptide *in vitro*. The success of these attempts, however, has been limited due to the difficulty of getting these large molecules across the blood-brain barrier. Until the present invention, diagnosis, treatment and prevention, of amyloid associated diseases has had little success.

The present invention comprises methods and compositions for diagnosing and treating diseases and processes mediated by the formation of abnormal amyloid deposition, such as amyloid plaques. One aspect of the invention comprises methods of administration of compositions comprising a fusion protein in a dosage sufficient to detect, and preferably dissolve, amyloid plaques. Another aspect comprising methods of making and using compositions comprising vaccine or antisera compositions. The present invention is useful in detecting and treating neurodegenerative diseases, such as Alzheimer's Disease. It is also useful in detecting and treating amyloid plaques in Lewy body dementia, Down's syndrome, hereditary cerebral hemorrhage with amyloidosis (Dutch type) and in the Guam Parkinson-Dementia complex and other amyloid-associated diseases.

An aspect of the invention comprises the construction and administration of fusion proteins comprised of 1) at least one segment of a binding region which binds to an epitope or fragment, preferably an epitope such as one located in ß-amyloid and 2) one or more segments comprising portions, fragments or whole proteins that are capable of crossing the blood brain barrier, such as one or more transferrin moieties. The segment comprising the binding region can be any binding partner that is capable of

attaching, binding or associating with an amyloid-associated moiety. For example, the variable region of an antibody, including the light or heavy chain or both, can be the binding region that is capable of binding an epitope found on an amyloid protein. In a particular aspect of the invention, the variable region of the light-chain of the 6C6 anti-β-amyloid mAb was sequenced and cloned with human transferrin to produce a molecular construct that expressed fusion proteins: V_L-Transferrin and V_L- $(Transferrin)_2$. The fusion proteins retained the property of the antibody 6C6 to solubilize ß-amyloid fibers and tangles. The addition of the transferrin moieties allows the fusion protein to cross the blood-brain barrier.

The present invention also comprises genes which code for these fusion proteins, expression vectors which contain these genes, microorganisms and cells transformed with these expression vectors as well as a process for the preparation of the genes, proteins, expression vectors and transformed cells and microorganisms.

The present invention further comprises other methods and compositions for preventing or treating diseases and processes associated with amyloidosis. A further aspect of the invention comprises the administration of compositions comprising a vaccine in a dosage sufficient to elicit an immune response against amyloid epitopes, such as proteins or peptides, particularly those found in plaques, tangles or other depositions. It also comprises the administration of compositions comprising antisera raised against amyloid epitopes or immungenic regions in a dosage sufficient to combat amyloidosis. This invention is particularly useful for diagnosing, preventing and treating neurodegenerative diseases associated with amyloid deposition, also referred to as amylodi-associated diseases, such as Alzheimer's, and other conditions associated with localized amyloidosis, such as Adult Onset Diabetes.

The present invention is also directed to methods of inhibiting production of Alzheimer-type amyloidosis in a mammal comprising

administering a pharmaceutically effective amount of a modified amyloid peptide capable of eliciting an immune response wherein the immune response prevents, hinders, or decreases the amount of amyloid deposition. The alleviation of symptoms related to amyloidosis followed by administration of derivaterized amyloid peptides, such as those described herein, results from stimulation of appropriate immune responses such that accumulation or formation of the amyloid aggregates is significantly altered, slowed or prevented.

Amyloid-associated conditions or diseases include, but are not limited to, Type 2 diabetes mellitus, amyloid A (reactive), secondary amyloidosis, familial mediterranean fever, familial amyloid nephropathy with urticaria and deafness (Muckle-wells Syndrome), amyloid lambda L-chain or amyloid kappa L-chain (idiopathic, myeloma or macroglobulinemia-associated) A beta 2M (chronic hemodialysis), ATTR (familial amyloid polyneuropathy (Portuguese, Japanese, Swedish), familial amyloid cardiomyopathy (Danish), isolated cardiac amyloid, (systemic senile amyloidosises), AIAPP or amylin insulinoma, atrial naturetic factor (isolated atrial amyloid), procalcitonin (medullary carcinoma of the thyroid), gelsolin (familial amyloidosis (Finnish)), cystatin C (hereditary cerebral hemorrhage with amyloidosis (Icelandic)), AApo-A-I (familial amyloidotic polyneuropathy - Iowa), AApo-A-II (accelerated senescence in mice), fibrinogen-associated amyloid; and Asor or Pr P-27 (scrapie, Creutzfeld Jacob disease, Gertsmann-Straussler-Scheinker syndrome, bovine spongiform encephalitis) or in cases of persons who are homozygous for the apolipoprotein E4 allele.

Fusion Proteins

One aspect of the present invention comprises methods and compositions comprising molecular constructs and the resulting expressed proteins or cells transformed by such constructs. Compositions comprising molecular constructs or the expression of such molecular constructs result in

compositions of fusion proteins of the present invention comprising fusion proteins having at least one segment that binds to a portion of amyloid, such as a peptide, protein, tangle or plaque; and at least one segment that is capable of crossing the blood-brain barrier. In particular, it is preferred that the segment that binds to amyloid is a binding region of an antibody or antibody fragment that binds to an epitope of amyloid, preferably an amyloid peptide or proteins. More particularly, it is preferred that the binding of the fusion protein to the epitope cause the dissolution of amyloid deposits, prevent deposition of amyloid or detect the presence of amyloid deposits.

Incorporation of genes or other nucleic acid segments, such as coding regions, into expression vectors can be effected using methods known to the skilled artisan. In this context, reference can be made to the textbooks of Maniatis et al. (*Molecular Cloning*, Cold Spring Harbor Laboratory, 1982) and Sambrook et al. (*Molecular Cloning-A Laboratory Manual*, 2nd. ed., Cold Spring Harbor Laboratory, 1989), the contents of both being herein incorporated by reference.

Methods for the expression of molecular constructs of fusion proteins in accordance with the invention are also known to the skilled artisan, and are described in detail in the aforementioned textbooks. The methods generally comprise the following steps:

a) transformation of a suitable host organism with an expression vector in which a gene or coding region is operatively bonded to an expression control sequence;

b) cultivation of the host organism under suitable growth conditions; and

c) extraction and isolation of the desired fusion protein from the host organism. Host organisms include, but are not limited to, gram-negative and gram-positive bacteria, for example *E. coli* and *B. subtilis* strains, yeast, insect cells, human and other animal cells.

A preferred method for producing compositions comprising fusion proteins using recombinant DNA techniques involves (1) sequencing the binding region segment of the fusion protein, for example, the variable region of a light chain of an antibody, (2) generating a synthetic gene for the expression of the variable region, (3) ligating the synthetic gene into a vector, such as the C-terminally His_6-tagged pet24d+ vector or pet36b+ vector bearing a C-terminal cellulose binding domain (CBD), (4) expressing this construct in *E. coli*, (5) fractioning the bacterial lysates, (6) purifying the appropriate fractions using Ni-loaded columns, (7) conducting a Western blot of the purified fraction to reveal the desired protein band; (8) amplifying the cDNA of the variable region of the antibody and the cDNA of human transferrin by PCR techniques, (9) inserting the cDNA of the variable region and the cDNA of human transferrin into a baculovirus vector, such as pBlueBacHIS2 B, (10) cloning the V_L-transferrin and V_L-$(transferrin)_2$ hybrids, (11) verifying the maintenance of the correct coding sequence by sequencing the inserts, (12) analyzing crude extracts of insect cell cultures to verify that they are positive for V_L-transferrin by SDS-PAGE, (13) performing Western blot analysis with an anti-His_6 to confirm these results, (14) purifying the constructs, (15) labeling the constructs with ^{111}In, (15) administering the constructs in an appropriate dosage with a suitable carrier, (16) performing external gamma-camera and radioactivity counting studies of the brain parenchyma and vasculature to quantify the constructs' capacity to cross the blood-brain barrier and to determine the presence of amyloid plaques.

The fusion protein compositions described herein have therapeutic utility, particularly in treatment of amyloid-associated diseases. Methods of treatment comprise administration of an effective amount of a composition comprising a fusion protein. The subject is then monitored for changes in amyloid plaques or cessation of symptoms. The methods of administration comprise one-time administration, sequential administrations or long-term bolus or continuous infusion administrations.

The fusion protein compositions are preferably administered to the mammal in a carrier. Suitable carriers and their formulations are described in Remington's Pharmaceutical Sciences, 16th ed., 1980, Mack Publishing Co., edited by Oslo et al. Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic. Examples of a pharmaceutically-acceptable carrier include saline, Ringer's solution and dextrose solution. The pH of the solution is preferably from about 5 to about 8, and more preferably from about 7 to about 7.5. Further carriers include sustained release preparations such as semipermeable matrices of solid hydrophobic polymers containing the fusion protein, which matrices are in the form of shaped articles, e.g., films, liposomes or microparticles. It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of protein being administered.

The fusion protein compositions can be administered to the subject, preferably an animal, mammal or human, by injection (e.g., intravenous, intraperitoneal, subcutaneous, intramuscular), or by other methods such as infusion that ensure its delivery to the bloodstream in an effective form. The compositions may also be administered by intratumoral, peritumoral, intralesional, or perilesional routes, to exert local as well as systemic therapeutic effects. Local or intravenous injection is preferred.

Effective dosages and schedules for administering the fusion protein compositions may be determined empirically, and making such determinations is within the skill in the art. Those skilled in the art will understand that the dosage of protein that must be administered will vary depending on, for example, the subject which will receive the composition, the route of administration, the particular type of fusion protein in the composition and other drugs being administered to the subject. A typical daily dosage of the fusion protein in the compositions of the present invention

range from about 1 µg/kg to up to 100 mg/kg of body weight or more per day, depending on the factors mentioned above.

The fusion protein compositions may also be administered to the subject in combination with effective amounts of one or more other therapeutic agents. It may be administered sequentially or concurrently with the one or more other therapeutic agents. The amounts of fusion protein compositions and therapeutic agents depend, for example, on what type of therapeutic agents are used, the condition being treated, and the scheduling and routes of administration. Following administration of such individual compositions or mixtures of compositions to the subject, the animal's physiological condition can be monitored in various ways well known to the skilled practitioner.

Fusion protein compositions may further be used in diagnostic assays for detecting specific antigens or as a nucleic acid probe for , e.g., detecting the presence or expression in specific cells, tissues, or serum, *in vitro* or *in vivo*. Various *in vitro* diagnostic assay techniques known in the art may be used, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases. See Zola, Monoclonal Antibodies: A Manual of Technicrues, CRC Press, Inc. (1987) pp. 147-158.

The molecular constructs or expressed proteins used in the present invention can be labeled with a detectable moiety. The detectable moiety preferably is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ^{3}H, ^{14}C, ^{32}P, ^{35}S, or ^{125}I, a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety may be employed.

In a further embodiment of the invention, there are provided articles of manufacture and kits containing compositions comprising fusion proteins that can be used, for instance, for therapeutic methods or other applications described above. The article of manufacture comprises a container with a label. Suitable containers include, for example, plates, slides, bottles, vials, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which includes an active agent that is effective for therapeutic or non-therapeutic applications, such as described above. The active agent in the composition can comprise the molecular construct or fusion protein. The label on the container indicates that the composition is used for a specific therapy other application, and may also indicate directions for either *in vivo* or *in vitro* use, such as those described above.

The kit of the invention will typically comprise the container described above and one or more other containers comprising materials desirable from a commercial and user standpoint, including buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

Vaccine Methods and Compositions

Additional methods and compositions of the present invention comprise compositions comprising at least one modified peptide, fragment or protein, preferably delivered in liposomal bilayers such as liposomes, that are used in methods to elicit an immune response. Preferably, these immune responses are able to overcome immune tolerance to "self" proteins. It is desirable to elicit such immune responses against amyloid peptides in order to treat or prevent amyloidosis. The products of the immune response are used to effect amyloid deposts, preferably in solubilizing amyloid aggregates. The products of the immune response, including but not limited to, antibodies, anitsera, stimulated cells and cellular factors, and antigens of modified

amyloid nature, peptides, fragments or proteins, comprise compositions of the present invention.

A preferred embodiment of the present invention comprises compositions of and methods of using a modified peptide, preferably, $A\beta_{1-16}$, to elicit an immune response. The immune response products or the immune response *in vivo* are used to dissolve or partially dissolve amyloid deposits. It is desirable to elicit an immune response against ß-amyloid 1- 16 ($A\beta_{1-16}$). A monoclonal antibody, 6C6, developed against the same epitope, the β-amyloid 1-16 peptide, is capable of solubilizing ß-amyloid fibers and tangles *in vitro*. This particular embodiment relates specifically to Alzheimer's disease and conditions associated with ß-amyloid aggregates, but this invention encompasses derivaterized amyloid peptides and their use to elicit immune responses capable of preventing or impeding conditions associated with amyloidosis in general and amyloid-associated diseases.

Preferred compositions comprise peptides that are modified by covalent binding of moieties, such as lipophillic moieties, such moieties are capable of presenting the peptide on the exterior of the delivery agent, such as anchoring the peptide in the lipid wall of a liposome. Preferred methods comprise administration into subjects, such as humans, mammals or other animals, for example, by injection.

Compositions comprising liposomes with modified peptides are used in methods of vaccination and compositions comprising antisera resulting from an immune response to an antigen described herein have therapeutic utility in treatment of amyloid-associated diseases, preferably AD. The compositions are preferably administered to the subject having an amyloid-associated disease. It will be apparent to those persons skilled in the art that carriers may be used, depending upon, for instance, the route of administration and concentration of vaccine or antisera compositions being administered. The vaccine or antisera compositions can be administered to the subject by injection (e.g., intravenous, intraperitoneal, subcutaneous, intramuscular), or

by other methods such as infusion to ensure delivery to the bloodstream is in an effective form. Additional routes of administration are included to exert local as well as systemic therapeutic effects.

Effective dosages and administration methods for delivery of the compositions comprising vaccines or antisera may be determined empirically and such determinations are within the skill of an artisan. Those skilled in the art will understand that the dosage required depends on the subject receiving the protein, the route of administration, the particular type of peptide antigen used and other substances being administered, among other considerations.

The vaccine or antisera compositions may also be administered to the subject animal in combination with effective amounts of one or more other therapeutic agents. They may be administered sequentially or concurrently with the one or more other therapeutic agents. The amounts of vaccine or antisera compositions and therapeutic agent depend on the type of therapeutic agents are used, the condition being treated, and the scheduling and routes of administration, among other considerations. Following administration of vaccine or antisera compositions to the subject animal, the animal's physiological condition is monitored in various ways well known to the skilled practitioner.

This invention is further illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof. Conversely, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims.

EXAMPLES

EXAMPLE 1: Protein sequence analysis of the 6C6 antibody light chain (V_L)

The monoclonal antibody 6C6 (Athena Neurosciences, San Francisco, CA) recognizes an epitope located in the 1-16 region of β-amyloid and is capable of solubilizing β-amyloid fibers and tangles in vitro. See Tamaoka et al. 1992, Proc. Natl. Acad. Sci, USA, 89, 1345-9.

Light and heavy chains of 6C6 were separated under reducing conditions in an agarose gel. An in-gel Lys-C-digestion with Trypsin was performed on the Kappa light chain using 1mg Lys-C overnight. Fractions were separated by reverse phase chromatography (HP-C90-1-IPLC 1 mm x 12.5 cm, DAD-detector). The mass of collected fractions was determined by mass spectrometry (MALDI-TOF). N-terminal sequencing was performed using a PE-Biosystems-sequencer (Procise ABI 492, Langen, Gemany). Other fragments were obtained by in-gel digests with the endoprotease V-9 cleaving C-terminal of Glu. Peptides were sequenced starting at the N-terminus. The sequence of the V_L chain is given in FIG. 1 and SEQ ID NO:1 and SEQ ID NO:2.

EXAMPLE 2: Gene synthesis and cloning of the V region of the light chain (V_L)

The amino acid sequence of the fragment V_L was converted into a nucleotide SEQ ID 1 and inserted into an appropriate vector system. The V_L gene fragment was synthesized using a PCR-based approach. The sequences of the sense and anti-sense strands were divided approximately into 25nt oligonucleotides at the 5'-end (external oligo) followed by four 50mers (internal oligo) in a gapless manner. Internal oligonucleotides were mixed and used as template in a PCR with the two external 5'-oligos as primers, in a

100-fold excess. PCR was performed in 50µl volume using 1 unit of a proof-reading polymerase (Ultma, Perkin-Elmer) with 2mM $MgCl_2$, buffers as supplied by the manufacturer and 25pmol of each external primer. The reaction started 2 minutes after incubation at 99° C in a Sanyo thermocycler by addition of the polymerase going through 30 cycles at 95° C for 2 minutes, 66° C for 2 minutes, and 72° C for 1 minute. The V_L gene sequence was verified by gel electrophoresis.

The PCR product was cleaved by *NeoI* and *XhoI*. The insert was prepared by agarose gel electrophoresis and ligated into a prepared pET24d+ vector or pET36b+ vector (Novagen), respectively. Initially the plasmid pET24d+ was used to insert the gene fragment (V_L) next to a hexa-histidine tag in order to detect and purify the fragment in subsequent experiments. Unfortunately, the anti-His-tag antibodies did not act sufficiently in the detection procedure showing high non-specific binding in Western blot analysis. Therefore, a second expression vector, pET36b+ containing a cellulose-binding domain (CBD) of a prokaryotic cellulase and several short peptide tags was used in order to purify the protein. The CBD included a propeptide acting as signal for the translocation of the CBD to bacterial periplasm. The plasmid pET24d+ containing V_L was named pETVL102; the plasmid pET36b+ with the V_L insert was named pETVL112. The maps of both vectors are given in FIG 2 and 3.

EXAMPLE 3: Expression in *E. Coli*

E Coli B21 (DE3)pLysS were transformed with the plasmid pETVL102 (pETVL112) and the empty vector pET24d+ (pET36b+) as a control. Overnight cultures were used to inoculate 50 ml of LB-medium containing 50µg/ml kanamycin and 30µg/ml chloramphenical. Cultures were grown at 37° C for 2 hours and agitated at 200 rpm. Expression was induced by the addition of IPTG (final concentration 0.5mM). 2ml samples were taken after 2 hours and 4 hours and centrifuged (5min, 1600 x g, 4°C). The pellets were washed with 1 ml of TrisHCl buffer (25mlM, pH = 7.5) and dissolved in 200 µl of lx SDS gel-loading buffer (RotiLoad 1, Carl Roth). The samples were sonicated, heated (5 minutes, 95° C) and analyzed on a 15% SDS-PAGE.

In a subsequent step, expression of the V_L-fragment in pETVL112 was repeated on a 2.5 L scale. The total soluble cell fractions were subjected to immobilized nickel affinity chromatography (Ni-1MAC) in a FPLC system. A column of Chelating Sepharose FF (Pharmacia Biotech) charged with Ni2+ ions was equilibrated with Buffer A (25mM TrisHC1, 0.1mM NaCl pH=7.5).

A 3 ml sample was loaded, followed by washing with Buffer A containing 20mM imidazol·Hel. Elution was accomplished with Buffer A containing 500mM imidazol·HCl and the fractions were analyzed in a Western blot.

The rest of the soluble cell extract of *E. Coli* pETVL112 was treated as described above and fractions around n°58 were pooled. This pool was subjected to a second affinity chromatography using a cellulose matrix. The CBD binds to cellulose in the presence of salt and is eluted either by pure water or by an organic solvent, e.g. ethylene glycol. The pool (20 ml) was forced through two columns (CBinD 900 Cartridges, Novagen). Then, the columns were washed with 10 ml wash buffer (20mM TrisHC1, 0.8M NaCl, pH=7.5) and 2 ml loading buffer (20mM TrisHCl, pH=7.5). Each column

was eluted with 1 ml 100% ethylene glycol. The flow-through, the washing solution and the eluate, was analyzed in Coomassie blue-stained SDS-PAGE and Western blot.

In sonicated cell fractions only the soluble fraction showed the specific 30kDa signal. It was concluded that the V_L-fragment might be very unstable even if fused to a stable moiety, and that it was not deposited as intracellular inclusion bodies, but processed correctly (unprocessed fusion protein has a calculated mass of about 34kDa) and translocated to the host's periplasm. For the verification of these results the expression of V_L in pETVL112 was repeated on a larger volume. The results were collected by affinity chromatography on a FPLC system. The absorption was monitored at 290 nm. The imidazol step gradient was used. The specific elution peak was seen at approximately 120ml total elution volume.

Eluate fractions were analyzed in a Western blot. 7.5 ml of fraction 8, fraction 25, fraction 33, and fraction 58 of the pETVL112 strain and the control (even-numbered lanes) were separated on a 12% SDS-PAGE, transferred to nitrocellulose, incubated with anti-CDB-antiserum, and assayed by protein A-peroxidase conjugate and chemoluminescence. The specific signal was seen in lane 7. Other signals are ambiguous due to a second antiserum that was used to detect the molecular weight marker (M).

The Coomassie blue-stained SDS-PAGE showed no band at the expected position. This suggests a very small amount of the fusion protein is present after this first chromatographic step relative to the total.

The rest of the soluble cell extract of *E. Coli* pETVL112 was treated similarly and a collection of fractions was forced through columns. Eluate fractions were analyzed by Western blot. The resulting eluate was almost without cross-reacting signals. On the Coomassie blue-stained gel a very faint signal at 30kDa was visible. See SEQ ID NO:3 and Figures 2(a) and (b).

EXAMPLE 4: Construction of V_L-human transferrin hybrids

Various V_L-transferrin hybrids were ligated and cloned into the baculovirus vector pBlueBacHIS2 B (4.9kb, Invitrogen) for expression in insect cells. The V_L fragment. (pETVL102) and cDNA for human transferrin (pUC18/hTF, human full length transferrin, 2.3kb in pUC18 vector, 2.7 kb, obtained from R.T.A. MacGillivray, Department of Biochemistry and Molecular Biology, Vancouver, Canada) were amplified by PCR. The introduction of stop codons and restriction sites at the end of the fragments were done using modified primers.

The following fragments containing either the cDNA of V_L or the cDNA of human transferrin, stop codons, and appropriate restriction sites were successfully amplified:

*Bam*H I- V_L-*Xho*I
Xho Ihtf-STOP-*Sal* I
Xho I-hTF-*Bgl* II
Bgl II-hTF-STOP-*Sal* I

Cleavage of the amplified DNA fragments by restriction enzymes allowed the ligation of the purified V_L and transferrin fragments in the following manner:

1. *Bam*H I-V_L-*Xho* I x *Xho* I-hTF-STOP-*Sal* 1
2. *Bam*H I-V_L-*Xho* x *Xho* I-hTF-*Bgl* II x *Bgl* II-hTF-STOP-*Sal* 1

Cleavage 1 yielded a fragment containing V_LTF which was ligated by the restriction site *Xho* 1 and ended by a stop codon.

In cleavage 2, V_L is connected with transferrin over *Xho* I followed by a second transferrin connected to the first over the restriction site *Bgl* II. The fragment ends with a stop codon.

The V-region of the light chain with one transferrin moiety (V_L-TF) as well as the V-region with two transferrin molecules (V_L-$(TF)_2$) were cloned into the multiple cloning site of the baculovirus vector pBlueBacHIS2 using the restriction sited *Bam*H 1 and *Sal* I. Insertion of these two selected recombinant clones were verified before sequence analysis.

Starting from pETVL102 the V_L was amplified by PCR using a DNA-polymerase with 3'->5' proofreading-activity. By using V_L-specific primers with 5'-terminal extensions, recognition sites for restriction enzymes (5'-end: *Bam*H I, 3'-end: *Sal* I) and a STOP-codon following the last amino acid of the V_L-fragment were added to the PCR-product.

The purified V_L-PCR product and the vector pBlueBacHIS2 B were cleaved with the restriction enzymes *Bam*H I and *Sal* I. The restricted vector was dephosphorylated using calf intestine phosphatase (CIP). The restriction products were identified by agarose gel electrophoresis before the V_L fragment was ligated with the CIP-treated pBlueBacHIS2 B vector. The T4 DNA-ligation and CIP treatment were performed as described by the manufacturer (MBI Fermentas). See FIG. 4(a) and (b).

EXAMPLE 5: Transformation, screening and DNA-sequencing of the subcloned V_L with the baculovirus vector pBlueBacHIS2 B

Competent *E. Coli* XL- 1 Blue MRF cells (Stratagene) were transformed by the ligation product pBlueBacHIS2 B containing the V_L-fragment by applying routine procedures (Sambrook et al., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, New York). After the transformation, six of the resulting clones were tested for the presence of the V_L insert.

The analysis of the single colonies was done by a PCR-based technique using the same V_L-specific primers, which were previously used for V_L-amplification. Verification of the construct was done by restriction cleavage and sequencing of the inserts.

The *Bam*H I/*Sal* I-restricted V_L-fragment was ligated with *Bam*H I/*Sal* I, digested, and CIP-treated pBlueBac HIS2 B vector.

After transformation, six of the resulting clones were tested for the presence of the V_L-insert. Analysis of single colonies shows that all six

clones contained the V_L-insert. Clone "V_L -1" was selected for further analysis by DNA-sequencing.

The maintenance of the correct coding sequence was verified by sequencing the inserts pBlueBacHIS B-V_L, pBlueBacHIS B-VL-TF and pBlueBacHIS B-V_L-TF_2. The maps of the pBlueBacHIS2 B containing the V_L-fragment and V_L-transferrin are shown in FIG. 4 (a) and (b).

EXAMPLE 6: Generation of the recombinant baculovirus and expression of the proteins V_L, V_L-TF and V_L-$(TF)_2$

Expression of the fused proteins V_L-TF and V_L-$(TF)_2$ as well as V_L was performed in *E. coli*. The proteins were analyzed in a Coomassie-stained gel. Three selected plasmids, pBlueBacHIS2 BVL-2, pBlueBacIIIS2 BVL-Tf-4 and pBlueBacIIIS2 B V_LL-TF2-5, were verified by DNA sequencing before the *in vivo* recombination of transfer vector and virus DNA was performed (Ba N-BlueTM Linear DNA, Invitrogen).

Sf9-cells were transfected by the plasmids pBlueBacHIS2 BVL-1, pBlueBacHIS2 BVL-TF-4 and pBlueBacHIS2 B V_L-TF2-5 as well as by acceptor-DNA (Bac-N-Blue TM-DNA, Invitrogen). The supernatants were collected after 3 and 5 days and tested in dilutions of 1:10 to 1:10,000 on agarose/X-gal-overlay plates. After 5 days of incubation, viruses were isolated from 8 plaques.

Sf9-cells were infected with these viruses (four samples per plaque) and supernatants were collected 4 days later (named P1-lysates) in order to isolate viral DNA. The DNA was characterized by PCR-technique.

EXAMPLE 7: Analysis of the fused proteins expressed by the baculovirus vector system

For the generation of lysates with a high titer of viruses, $3x10^7$ Sf9-cells were infected with the PI lysates of V_L, V_L-TF and V_L-TF_2 with a m.o.i

(multiplicity of infection) of approximately 0.1. After 3 days the supernatants were collected and their titers determined by limited dilutions. The proteins were isolated and characterized in a Coomassie-stained 10% PAGE-gel.

The PCR with V_L showed the expected fragments as well as V_L-TF in a Coomassie-stained gel. The amplification products of V_L-TF_2 (light chain with 2 transferrin moieties) seem to have lost one of the transferrin residues showing a length of the product of V_L-TF or had lost an even longer fragment. Because of the instability of V_L-TF_2 , the virus DNA of four other clones was analyzed by PCR. Unfortunately, no V_L-TF_2 with the correct length was detected.

A virus titer of $2x10^8$ pfu/ml (plaque forming units) for V_L-1 (26 ml), $1x10^8$ pfu/ml for V_L-TF4 (26 ml), and $1x10^8$ for V_L-TF_2 (14 ml) was obtained from the three selected baculovirus clones V_L, V_L-TF, and V_L-TF_2. From the V_L- and V_L-IF-infected cells, proteins with the expected molecular weights of 15kD and 80kD could be extracted. The extract of V_L-TF_2 showed two proteins with the molecular weight of 80kD and about 105kD, both not the relevant protein of V_L fused to two transferrin molecules. Western blot analysis of crude extract of insect cells cultures containing V_L and V_L-transferrin performed with an anti-His_6 confirmed these results.

Example 8: In vivo Administration

High grade purification (>95%) of the constructs is performed. A sufficient amount of V_L and V_LTF is obtained, and the constructs are labeled with ^{111}In. External γ-camera and radioactivity counting studies of the brain parenchyma and vasculature allows the quantitation of the constructs' capacity to cross the blood-brain barrier.

Example 9: Preparation of palmitoylated lysines for solid phase peptide synthesis

The following methods were used to produce vaccine compositions. Palmitoylated lysines were appended to both ends of the Aβ 1-16 peptide to anchor the ß-amyloid l- 16 ($A\beta_{1-16}$) peptide in the liposome bilayer. See FIG. 5.

Two strategies were adopted and two tetrapalmitoylpeptides were synthesized by means of an Applied Biosystems peptide synthesizer: 1) the N-terminus has two palmitoyl residues, (i.e. α,ε-dipalmitoyllysine) while at the C-terminus two a-palmitoyllysines were inserted sequentially; 2) four a-palmitoyllysines are inserted, two at each end. For the automated solid phase synthesis of the peptide the first amino acid, a-palmitoyllysine, was N-protected and anchored on the 4-alkoxybenzyl alcohol resin by means of an ester linkage. Fluorenylmethoxy-carbonyl (Fmoc) group was preferred as the a-amine protecting group for lysine and its derivatives. Methods for making such building blocks are known. See FIG 6 a and b.

Example 10: Anchoring of FmocLys(PAL) on the Alkoxybenzyl alcohol Resin

FmocLys(Pal)OH (from BACHEM) was reacted with the alkoxybenzyl alcohol resin (from BACHEM) in the presence of dieyclohexylcarbodiimide (DCC, Aldrich) and dimethyl-aminopyridine (DMAP, Aldrich) in dry, freshly distilled methylene chloride according to the procedure optimized by G. Lu et al., *J. Org. Chem.*, 46, 3433 (1981). After stirring for three hours at room temperature, the reaction mixture was filtered and washed thoroughly ten times with dry methylene chloride. To ensure complete reaction, the obtained resin was reacted once more with a fresh portion of FmocLys(Pal)OH, in the presence of DCC and DMAP in dry methylene chloride at room temperature overnight. The next day, after

filtration and washing with methylene chloride the resin was dried in vacuum. The FT-IR spectrum of the product shows the expected bands for the ester linkage at 1720 cm^{-1}, for the NH group at 3327 cm^{-1} and for the amide carbonyl group 1626 cm^{-1}. All these bands are absent in the FT-IR spectrum of the starting alkoxybenzyl alcohol resin. The final quantity was 227 mg with a loading of about 0.5 mmol/g. See FIG 6 (c).

Example 11: Solid phase synthesis

To the resin, the second palmitoylated lysine was added by means of FmocLys(Pal)OH after deprotection (removal of the Fmoc group). Then 16 cycles of synthesis were performed for the β-amyloid. For test purposes, a small quantity of this peptide was then cleaved from the resin and investigated by electrospray mass spectrometry (ES-MS). The crude mixture shows the presence of three peptides, the major component being the desired one having peaks at 896.9, 673,0 and 538.0 Da corresponding to the +3, +4 and +5, respectively charged molecular ions. The molecular ion is absent in the spectrum, but can be inferred from this series to be at 2687.86 Da. The other two components correspond to peptides having one or two palmitoylated lysines less (367 Da mass difference) with molecular ions inferred at 2321.43 and 1953.87 Da. This indicates that the coupling of the FmocLys(Pal)OH was incomplete.

α,ε-dipalmitoyllysine was coupled to the rest of the uncleaved resin. Even prolonged coupling times (overnight at room temperature) left unreacted material (ninhydrin test) due to the low solubility in DMF of the dipalmitoylated lysine. After cleavage from the resin, the ES-MS shows that the desired tetrapalmitoylpeptide (M+ = 3292.43) is present only in about 20 %.

In a second run, to avoid the sluggish coupling with α,ε-dipalmitoyllysine, after the 16 cycles which apprehended the B-amyloid

residues to the first two palmitoyllysines were completed, two sequential palmitoyllisines were inserted at the end, the coupling being performed twice for each. The ES-MS spectrum after cleavage from the resin (with TFA) shows the desired peaks of the tetrapalmitoylpeptide as the M4+ at 855.4 Da and M5+ at 05.4 corresponding to a molecular ion of 3421.65. This peptide amounts to about 35 % or the mixture, the main component being a peptide having two Lys(Pal)-residues less (M+ at 2688.58 Da). A minor component has an extra lysine missing (M+ at 2560.42 Da).

The mixture or peptides was composed of the desired tetrapalmitoylated peptide and peptides lacking palmitoyl residues, thus making the double insertion into liposomes improbable. Therefore, we decided to use the mixtures for liposome preparation.

Example 12: Preparation of α,ε-dipalmitoyllysine

Direct palmitoylation or lysine with palmitoyl chloride in a Schotten-Baumann reaction with aqueous sodium hydroxide, on a 20 g scale led to a material which contained appreciable amounts of palmitic acid and which could not be separated from the desired a,e dipalmitoyllysine. An indirect method had to be adopted following the procedures of H. Kiwada, et al., *Chem. Pharm. Bull.*, 35, 2935-39 (1987); Y. Lapidat, et al., *J. Lipid Res.*, 9, 142-44 (1967).

The palmitoylester of N-hydroxysuccinimide was synthesized first from palmitic acid (Fluka), N-hydroxysuccinimide (Aldrich) in the presence of DCC (Aldrich) in ethyl acetate in 77% yield. This activated ester was subsequently reacted with the sodium salt of lysine in aqueous tetrahydrofurane. The crude product obtained after filtration and washing with water still contained some unreacted activated ester as put into evidence by proton NMR and FAB (Fast Atom Bombardment)-mass spectra. Recrystallization from chloroform provided a pure sample of 640 mg. Its

FAB-mass spectrum shows the expected peak at 623 Da (MH+) and absence of the peak for the activated ester (354 Da). See FIG. 7 and FIG. 8.

Example 13: Reconstitution of the palmitoylated peptides in liposomes

Liposomes with Lipid A were used as adjuvants in an attempt to break the mouse iminune tolerance to Aβ 1-16. They were prepared by mixing dimyristoyl-phosphatidylcholine, dimyristoylphosphatidyl-glycerol, and cholesterol (Avanti Polar Lipids, Alabaster, AI, USA) in the molar ratios 0.9,0.1:0.7. Monophosphoryi lipid A, a strong immunomodulator, (IASL Biologicals, Campbell, CA, USA) was added at a concentration of 40 mg per mmole of phospholipids. Tosi et al., Biochem. Mophys. Res, Com., 212, 494-500 (1995). The palmitoylated peptides were added at a molar ratio to phospholipids of 1:100 and 1:200. Solvents were evaporated. The resultant film, after hydration with sterile phosphate buffer saline (PBS, pH 7.4) with a final phospholipid concentration 4 mM, was further homogenized by orbital shaking. The liposome suspension was mixed with sterile Alum 15 minutes before injection (9:1 vol:vol, Rehydrogel, HYA, Rebeis Inc, Berkley Heights, NJ).

Example 14: Immunization of mice

Eight BALB/c mice (Charles River Laboratories, Wilmington, MA, USA) were immunized by 6 i.p. innoculations at two week intervals with 200 µL of the palmitoylated peptide-liposome/Alum suspension. One group of 3 mice was immunized according to the same protocol with PBS/Alum only. Another group of 3 mice were immunized according to this protocol, with 4 mM phospholipids in PBS and Alum, without palmitoylated Aβ 1-16. Blood was collected from the tail vein 4 days after injection. The collected blood (10-30 µl) was diluted immediately with 10µl PBS and 5µl heparin. The

samples were centrifuged, the serum was removed and tested in an ELISA assay.

A second group of 19 transgenic mice (NORBA, Hoechst Marion Roussel, Bridgewater, NJ) of different ages, constitutively present β-amyloid plaques on their pancreas. They were immunized using the same protocol described above. Before sacrificing, the immunized NORBA mice received a 5th immunization. Blood was collected and assayed for anti-β-amyloid antibodies in an ELISA assay.

Further, 12 B57131/6-mice were immunized as described before. In this group liposomes and palmitoylated β-amyloid (1-16), liposomes; mixed with scrambled β-amyloid (42-1) and liposomes were used for injection. Alum was added as before. As an additional control 3 mice were immunized with liposomes and palmitoylated β-amyloid (1-16), but without Alum. Blood was collected and tested as described.

Example 15: ELISA (Enzyme-Linked Immunosorbent Assay) Experiments

Microtiter plates were coated with 50 µl of β-amyloid 1-28 solution (1mg/ml) overnight at 40° C. Blocking of the wells by 200 µl BSA/PBS (0.5% BSA) for two hours at 37° C followed before washing with 200 µl of PBS/0.005% Tween 20.

Various dilutions of serum (1:100-1:100,000) were incubated for 2 hours at 370 C. The plates were then washed twice with 200 µL of PBS/0.005% Tween 20 before 50 µl of a goat-anti-mouse-antibody (alkaline phosphatase conjugated) in a 1:30,000 dilution was added. After 2 hours at 37° C the wells were washed as described above. Then, 100 µl of the substrate (PNPP, paranitrophenyl phosphate, 1 tablet in 5 ml distilled water) was added and absorption was measured at 405 nm by an ELISA reader 30 - 60 min later.

After the third injection with liposomes/palmitoylated β-amyloid (1-16), ELISA assays showed a significant immune response in the vaccinated Balb/c mice. The antibodies were specific to the injected antigen. The OD_{405} of 1:5000 - dilutions of the collected sera were 10 to 20 fold higher than those of controls which were dilutions of untreated mice. Thus, this immunization procedure elicited an immune response against Aβ 1-16 in mice. No immune response was detected in the mice having received control immunizations.

The same immunization protocol was used for inoculation of NORBA transgenic mice to study binding to and possibly dissolving of β-amyloid-plaques on their pancreases in vivo. 19 transgenic mice (NORBA, Hoechst Marion Roussel, Bridgewater, NJ) of different ages, constitutively present β-amyloidplaques on their pancreas, were immunized. Before sacrificing, the immunized NORBA mice received a seventh immunization. Blood was collected and assayed for anti-β-amyloid antibodies in an ELISA assay. In 1:5,000 dilutions of the sera the OD_{405} was 10 fold higher than in controls. The control NORBA mice did not show any anti-Aβ antibodies in their sera. The 3rd group of immunized C57B1/6 mice receiving Aβ 1-16 as well as the scrambled Aβ 42-1 and as a control, liposomes without a peptide reached a tenfold higher titer in a 1:10,000-dilution of the sera versus control. See Tables 1 and 2.

Table 1. ELISA of sera of Immunized Balb/c and C57B1/6 Mice

Antigen	Palm ß-Amyloid (1-16)/ liposomes, lipid A in C57Bl/6	Scrambled ß-Amyloid (42-1)/ liposomes, lipid A	Liposomes, lipid A	Palm ß-Amyloid (L-16)/ liposomes, lipid A in Balb/c	Untreated animal Balb/c
10000*	0.16	0.06	0.011	-	-
5000	0.22	0.01	0.012	0.22	0.01
2500	0.46	0.024	0.060	0.30	0.04
1000	0.61	0.011	0.041	0.31	0.03
500	0.99	0.044	0.039	0.46	0.04
100	-	-	-	0.60	0.03

OD_{405} Dilution: 1-* Secondary antibody: alkaline phosphatase conjugated goat anti mouse antibody. Substrate (PNPP)- p-nitrophenyl phosphate. Assay was performed after the 3. (C57BV6) and after the 4.

(Balb/c) booster injection. For each antigen/adjuvants 3 animals were injected.

Table 2: Immune response in mice inoculated with palmitoylated ß-amyloid (1-16), scrambled ß-amyloid (42-1) in combination with liposomes/lipid A and Alum and liposomes without amyloid.

Antigen	palm.-A_β 1- 16	A_β 42-1	Phospholipids
number of C57Bl/6 mice with a pos. titer in 1:10000 dilution of sera	2/3 after 5 Injections	0/3	0/3
number of Balb/c mice with a positive titer in 1:5000 dilution of sera	3/3 after 4 injections		0/3
number of transgenic NORBA mice with a pos. titer in 1:5000 dilution of sera	7/9 (Aventis) after 7 injections		

*OD 405 was tenfold of the control (only phospholipids)

Example 16: Pathology investigation of the immunized C57Bl/6 mice

The immunized C57Bl/6 mice underwent a pathological investigation after the immunization procedure was finished. Sections of kidney, liver, heart, bone marrow, pancreas, spleen and brain were analyzed.

Example 17: Immunohistochemical investigation of the pancreas of vaccinated NORBA mice.

23 NORBA mice of different ages containing β-amyloid-positive and β-amyloid-negative animals were immunized with palmitoylated Aβ (1-16) reconstituted in liposomes, as described (19 mice) or with liposomes only (4 mice). These animals have the β-amyloid plaques on the pancreas instead of

brain. The vaccinated mice were sacrificed after the 7th injection and their pancreases were collected and preserved in formalin. The preserved pancreas pieces were soaked in sucrose solution to prepare them for frozen sections. The thin sections obtained were analyzed by Thioflavin T, a fluorescence dye specific for B-amyloid aggregates staining (Vassar and Culling. 1959), to detect B-amyloid on the surface of the pancreas. The sections were analyzed also using a FITC-labeled anti-β-amyloid antibody (Accurate Chemical Co., Westbury. CT). The binding of the mouse "autoantibodies" to the β-amyloid plaques was assayed with a rabbit- antimouse IgG, FITC- labeled.

A histological study of Thioflavin T stained sections of pancreases from NORBA transgenic mice, vaccinated with palmitoylated Aβ (1-16) reconstituted in liposomes was performed. The stained pancreatic tissue of a B-amyloid negative animal showed a diffuse, weak, background fluorescence with some bright spots which represented vessels. The acinar cells were completely dark. An 18-month old-animal with fully developed B-amyloid plaques showed intense fluorescence throughout the acinar cell fields. There was also very bright fluorescent areas suggesting larger blood vessels. A 18-month old mouse with fully developed B-amyloid plaques, which was vaccinated and examined 4 months after first inoculation, showed focal (patchy) areas of fluorescence among acinar cells, but predominantly many patches of non-fluorescent acinar cells. The results showed that the vaccination either disintegrated β-amyloid plaques or reversed their deposition.

Quantitative evaluation of the average fluorescence intensity in each section stained with Thioflavin using a luminosity analysis software, indicated that the pancreas sections from 19 months old NORBA vaccinated animals showed less than 25% of the high intensity fluorescence of the same animals, unvaccinated. A sampling of these quantitations is shown in Table 3.

Table 3: Percentage of fluorescence intensities in Thioflavin-stained pancreas sections of NORBA mice, vaccinated and unvaccinated.

Animal	High intensity %	Medium Intensity %	Low intensity %
Control Aß⁻ mouse	0	11.65 +/-1.6	88.5+/- 9.1
Aß⁺,18-month old unvaccinated mouse	3.271-+/- 0.3	34.62 +/- 3.5	62.11 +/- 6.1
Aß⁺, 18-month old, vaccinated mouse	0.74 +/- 0.07	34.63 +/- 3.5	64.63 +/. 6.4

Each value is the average of 5 countings. It appears that vaccination reduces dramatically (by more than 70%) the high intensity fluorescence in the Thioflavine-stained pancreas sections of the NORBA transgenic mice with fully developed ß-amyloid plaques. The medium and low intensity fluorescence, which is unspecific since it is detected also in the negative controls, remains unchanged upon vaccination, There is no high intensity fluorescence, in the negative controls.

In *vitro* experiments were conducted to investigate the mechanism by which the "autoantibodies" disintegrate the plaques. β-amyloid aggregates using the sequence Aβ (1-28) were made by incubating for 7 days at 37° C in phosphate buffered saline (PBS) at pH=7.1. This is the sequence used for the immunization of the mice. These deposits were detectable by staining with Thioflavin T ("Th T"). Th T stains amyloid-like deposits and exhibits enhanced fluorescence emission at 482 nm and a new excitation peak at 450 nm when added to a suspension of aggregated β-sheet preparations (Solomon et al.(1997) PNAS 94, 4109- 4112). Figure 7 shows comparatively the disaggregation of β amyloid fibers by the 6C6 mAB and by sera from mice immunized with palmitoylated Aβ (1-16) reconstituted in liposomes.

It appears that both antibodies have solubilizing properties, but the monoclonal antibodies has a significantly stronger effect than the serum from the immunized mice, which contain polyclonal antibodies.

Any patents or other publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned and inherent within. The present examples along with the methods, procedures, treatments, compositions, and specific compounds described herein are presently representative of preferred embodiments, and are not intended as limitations on the scope of the invention. Those skilled in the art will know or will be able to ascertain many equivalents to the specific embodiments of the invention described in the examples above using only routine experimentation. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

CLAIMS

What is claimed is:

1. A molecular construct comprising, an expression vector encoding a fusion protein comprising at least one segment comprising a binding region which binds to an epitope or fragment, and at least one or more segments comprising portions, fragments or whole proteins that are capable of crossing the blood brain barrier.

2. The composition of Claim 1, wherein the at least one or more segments capable of crossing the blood-brain barrier is transferrin.

3. The composition of Claim 1, wherein the at least one or more segments comprising a binding region is an antibody or antibody fragment.

4. The composition of Claim 1, wherein the at least one or more segments comprising a binding region is an antibody or antibody fragment and the at least one or more segments capable of crossing the blood-brain barrier is transferrin.

5. A composition comprising, a fusion protein comprising at least one segment comprising a binding region which binds to an epitope or fragment, and at least one or more segments comprising portions, fragments or whole proteins that are capable of crossing the blood brain barrier.

6. The composition of Claim 5, wherein the at least one or more segments capable of crossing the blood-brain barrier is transferrin.

7. The composition of Claim 5, wherein the at least one or more segments comprising a binding region is an antibody or antibody fragment.

8. The composition of Claim 5, wherein the at least one or more segments comprising a binding region is an antibody or antibody fragment and the at least one or more segments capable of crossing the blood-brain barrier is transferrin.

9. The composition of Claim 5, wherein the fusion protein is capable of altering amyloid deposition in a human or animal.

10. A composition comprising at least one modified peptide, fragment or protein anchored in a liposomal bilayers.

11. The composition of Claim 10, wherein the at least one modified peptide, fragment or protein is an amyloid peptide, fragment or protein.

12. The composition of Claim 10, wherein the liposomal bilayer is a liposome.

13. A method of treating amyloid-associated diseases comprising, administering a pharmaceutically effective amount of the composition of Claim 5.

14. The method of Claim 12, wherein the amyloid-associated disease comprises Alzheimer's disease, Type 2 diabetes mellitus, amyloid A (reactive), secondary amyloidosis, familial mediterranean fever, familial amyloid nephropathy with urticaria and deafness (Muckle-wells Syndrome), amyloid lambda L-chain or amyloid kappa L-chain (idiopathic, myeloma or macroglobulinemia-associated) A beta 2M (chronic hemodialysis), ATTR (familial amyloid polyneuropathy (Portuguese, Japanese, Swedish), familial amyloid cardiomyopathy (Danish), isolated cardiac amyloid, (systemic senile amyloidosises), AIAPP or amylin insulinoma, atrial naturetic factor (isolated atrial amyloid), procalcitonin (medullary carcinoma of the thyroid), gelsolin (familial amyloidosis (Finnish)), cystatin C (hereditary cerebral hemorrhage with amyloidosis (Icelandic)), AApo-A-I (familial amyloidotic polyneuropathy - Iowa), AApo-A-II (accelerated senescence in mice), fibrinogen-associated amyloid; and Asor or Pr P-27 (scrapie, Creutzfeld Jacob disease, Gertsmann-Straussler-Scheinker syndrome, bovine spongiform encephalitis) and persons who are homozygous for the apolipoprotein E4 allele.

15. A method of treating amyloid-associated diseases comprising, administering a pharmaceutically effective amount of the composition of Claim 10.

16. The method of Claim 14, wherein the at least one modified peptide, fragment or protein is palmitoylated β-amyloid$_{1-16}$ peptide.

17. The method of Claim 15, wherein the at least one modified peptide, fragment or protein is adminstered in a liposomal bilayer.

18. The method of Claim 14, wherein the amyloid-associated diseases comprises Alzheimer's disease, Type 2 diabetes mellitus, amyloid A (reactive), secondary amyloidosis, familial mediterranean fever, familial amyloid nephropathy with urticaria and deafness (Muckle-wells Syndrome), amyloid lambda L-chain or amyloid kappa L-chain (idiopathic, myeloma or macroglobulinemia-associated) A beta 2M (chronic hemodialysis), ATTR (familial amyloid polyneuropathy (Portuguese, Japanese, Swedish), familial amyloid cardiomyopathy (Danish), isolated cardiac amyloid, (systemic senile amyloidosises), AIAPP or amylin insulinoma, atrial naturetic factor (isolated atrial amyloid), procalcitonin (medullary carcinoma of the thyroid), gelsolin (familial amyloidosis (Finnish)), cystatin C (hereditary cerebral hemorrhage with amyloidosis (Icelandic)), AApo-A-I (familial amyloidotic polyneuropathy - Iowa), AApo-A-II (accelerated senescence in mice), fibrinogen-associated amyloid; and Asor or Pr P-27 (scrapie, Creutzfeld Jacob disease, Gertsmann-Straussler-Scheinker syndrome, bovine spongiform encephalitis) and persons who are homozygous for the apolipoprotein B4 allele.

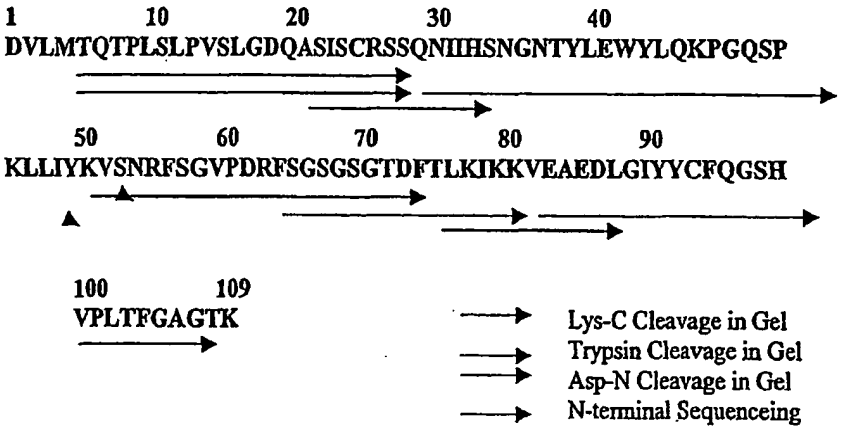
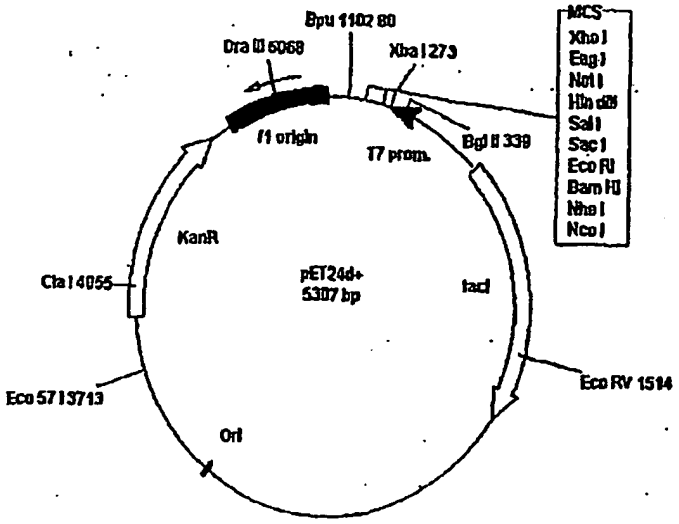

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50 60 70 80 90
KLLIYKVSNRFSGVPDRFSGSGSGTDFTLKIKKVEAEDLGIYYCFQGSH
100 109
VPLTFGAGTK
Lys-C Cleavage in Gel
Trypsin Cleavage in Gel
Asp-N Cleavage in Gel
N-terminal Sequenceing

FIG. 1



(a)

Bgl II T7 promoter Xba I
rba
AGATCTGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAAT
AAT

TTTGTTTACTTTAAGAAGGAGA

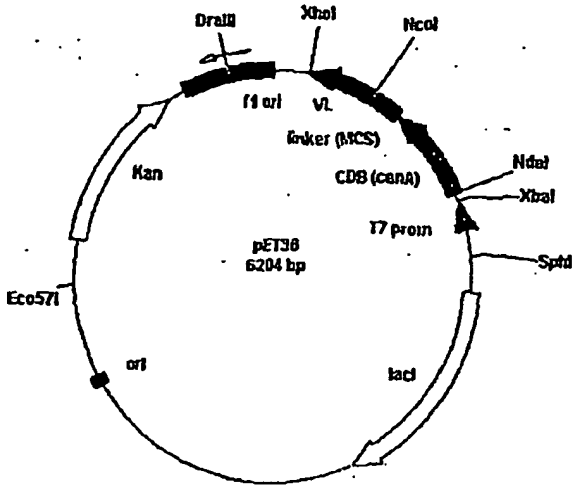
NcoI NheI BamHI EcoRI SacI SalI HindIII
NotI
XhoI

TATACCATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGC
C
GCACTCGAGCACCACCACCACCACCACTGA
T7 - Tag

His - Tag

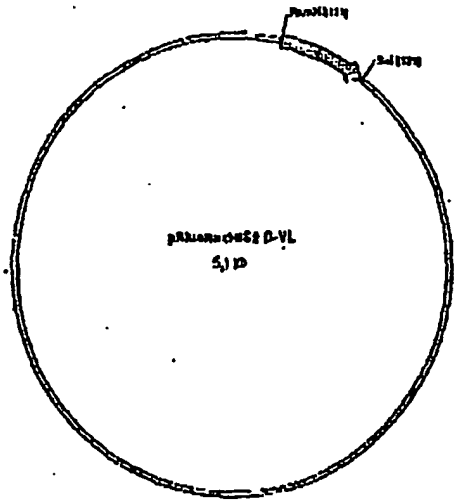
(b)

FIG. 2

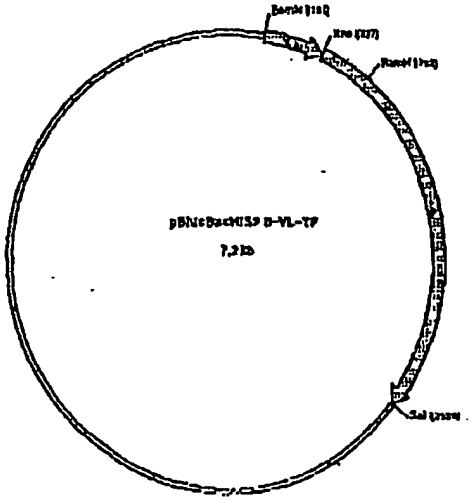


pETVL112 = pET36 + VL

FIG. 3



(a)



(b)

FIG. 4

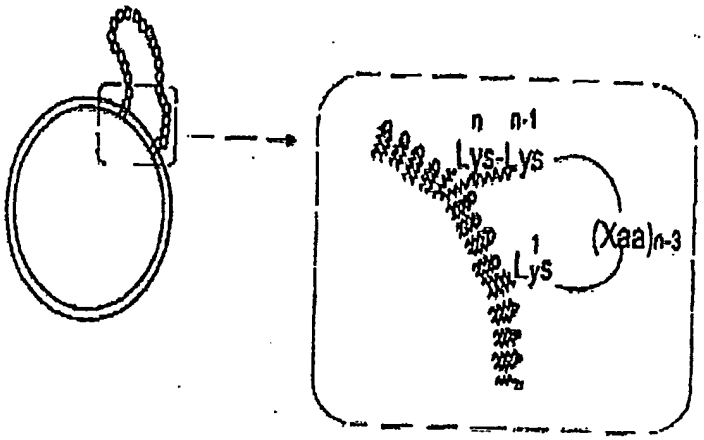
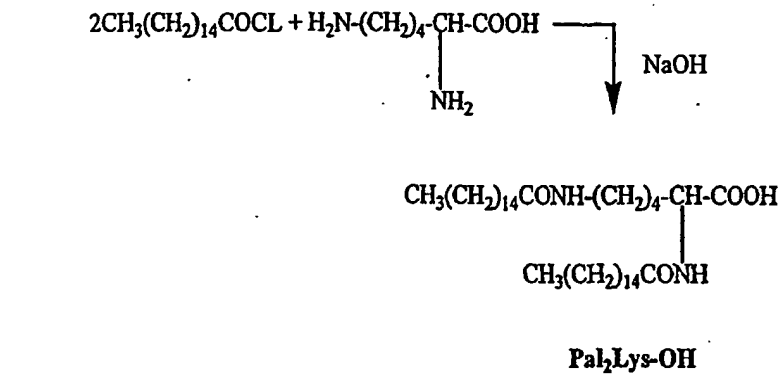
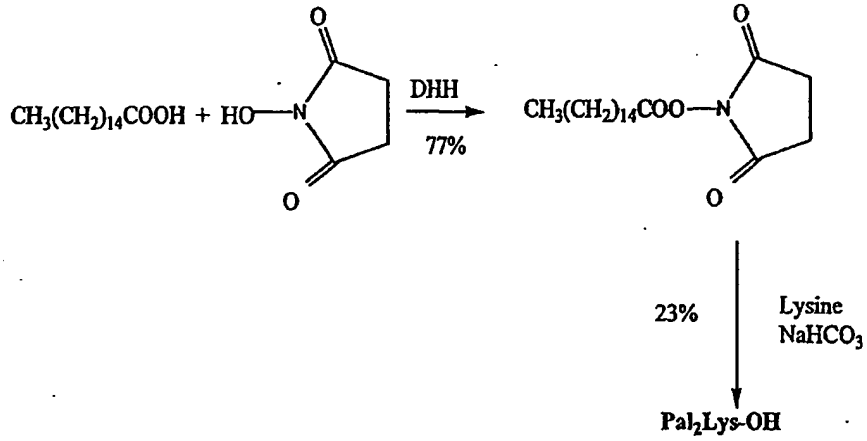


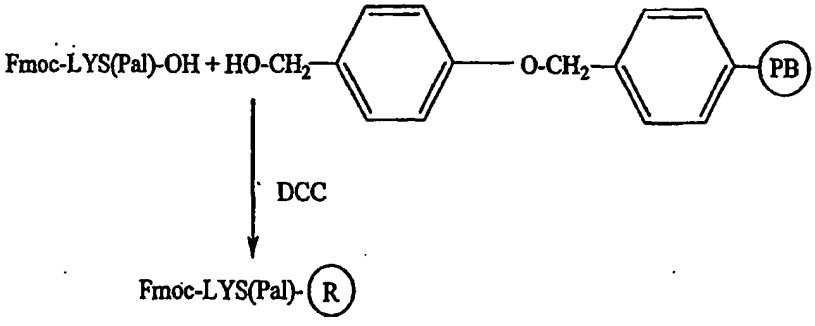
FIG. 5


2CH3(CH2)14COCL + H2N-(CH2)4-CH-COOH
NH2
NaOH
CH3(CH2)14CONH-(CH2)4-CH-COOH
CH3(CH2)14CONH
Pal2Lys-OH

a.


CH3(CH2)14COOH + HO-N
DHH
77%
CH3(CH2)14COO-N
23%
Lysine
NaHCO3
Pal2Lys-OH

b.


Fmoc-LYS(Pal)-OH + HO-CH2
O-CH2
PB
DCC
Fmoc-LYS(Pal)-
R

c.

FIG. 6

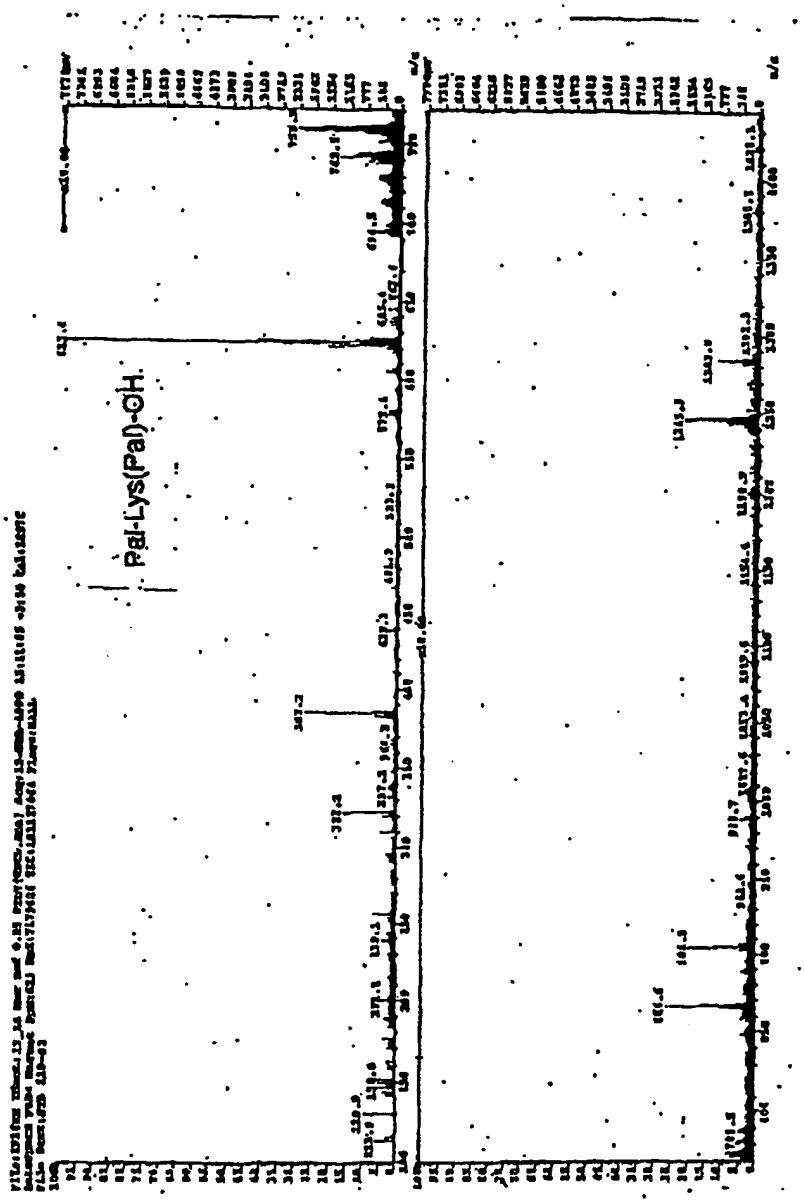
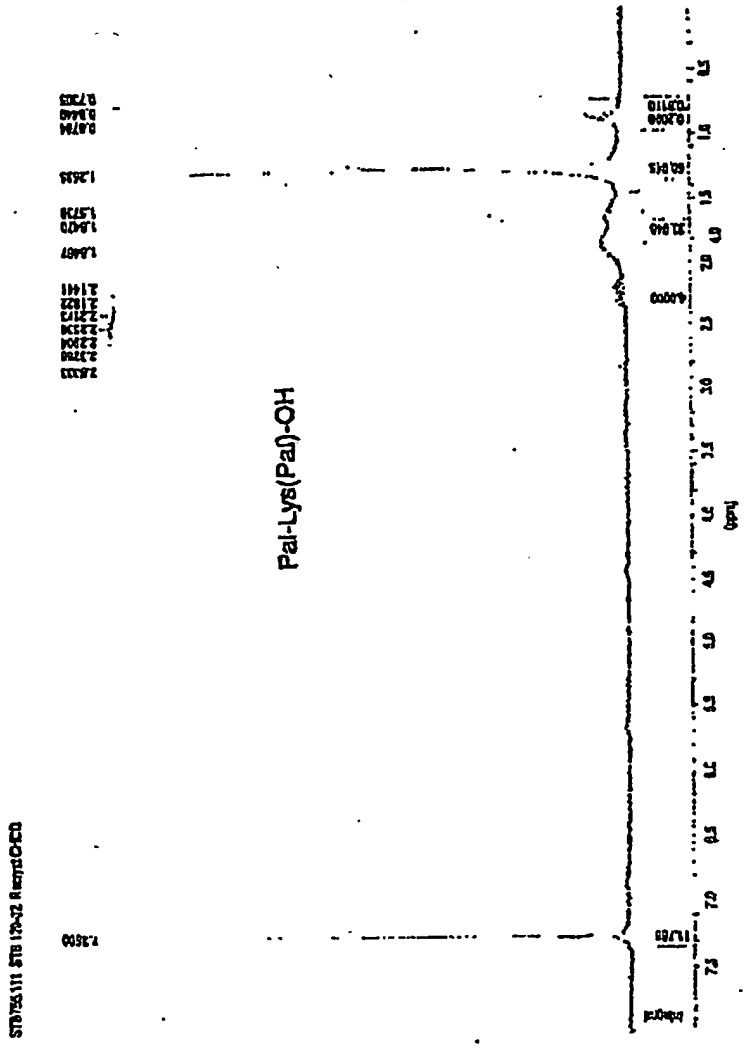


FIG.7


Pal-Lys(Pal)-OH
ppm

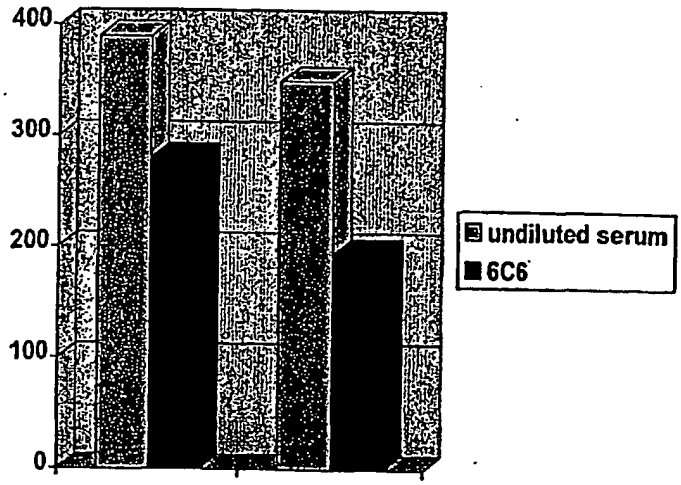


FIG. 9

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<151> 2000-12-12

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1               5                  10                  15

ggc gat cag gcg agt att tct tgc cgt tcc agc cag aac att atc cat       96
Gly Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Asn Ile Ile His
            20                  25                  30

agc aac ggc aat acc tat ctg gaa tgg tac ctg cag aaa ccc ggc cag      144
Ser Asn Gly Asn Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln
        35                  40                  45

agc ccg aaa ctg ctg att tat aaa gtg agc aac cgc ttt agc ggt gtg      192
Ser Pro Lys Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val
    50                  55                  60

ccg gat cgt ttt agc ggc agt ggt agc ggc acc gat ttt acc ctg aaa      240
Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys
65                  70                  75                  80

att aaa aag gtg gaa gcg gaa gat ctg ggc att tat tat tgc ttt caa      288
Ile Lys Lys Val Glu Ala Glu Asp Leu Gly Ile Tyr Tyr Cys Phe Gln
                85                  90                  95

ggt agc cat gtg ccg ctg acc ttt ggc gcg ggc acc aaa ctc gag          333
Gly Ser His Val Pro Leu Thr Phe Gly Ala Gly Thr Lys Leu Glu
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<211> 111

<212> PRT

<213> Murinae gen. sp.

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1               5                  10                  15

Gly Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Asn Ile Ile His
            20                  25                  30
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Ser Asn Gly Asn Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln
35 40 45

Ser Pro Lys Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val
50 55 60

Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys
65 70 75 80

Ile Lys Lys Val Glu Ala Glu Asp Leu Gly Ile Tyr Tyr Cys Phe Gln
85 90 95

Gly Ser His Val Pro Leu Thr Phe Gly Ala Gly Thr Lys Leu Glu
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ggtggacagc aaatgggtcg gatccgaatt cgagctccgt cgacaagctt gcggccgcac 180

tcgagcacca ccaccaccac caccactga 209

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